Rhizosphere is a hub for key bacterial species that regulate the soil structure and plant growth. Bacterial communities which reside in the rhizosphere possess plant growth-promoting traits that can aid in effective establishment of vegetation in barren lands and contaminated areas.

5.1 Collection of samples

Root rhizosphere is considered to be the reservoir for native PGPR strains as the microbes residing in them get adapted to the endemic soils conditions (Bergottini, 2015). Abou-Shanab et al. (2007) stated that microorganisms isolated from heavy metal contaminated soils possess the ability to withstand against heavy metal pollutants. Considering the above fact an attempt has been made in the present investigation to isolate efficient PGPR from tailing dam of Zawar mines, Udaipur, Rajasthan (India). A total of 41 soil samples were collected from rhizosphere of plants namely *Tridax*, *Calotropis* and *Acacia* species growing in tailing dam of Zawar mines. The samples were stored at 4°C and were processed for the isolation of bacteria within 24 h of sample collection.

5.2 Isolation of phosphate solubilizing bacteria

In the present study, a total of 265 bacterial isolates were recovered from 41 rhizospheric soil samples on Pikovskaya (PVK) agar containing 0.5% tri-calcium phosphate (TCP). Out of 265, 96 bacterial isolates produced halo zone of clearance around their colonies on PVK agar demonstrating the phosphate solubilization activity. Kloepper and Schroth (1981) reported the presence of large number of microorganisms in rhizosphere. They explained that the plant roots follow several mechanisms like release of root exudates, organic compounds, competition for nutrients and provides surface for adhesion which influence the rhizospheric soil microbes and increases the availability of bacteria near plant roots. This may be the probable reason for recovery of large number of isolates.
from rhizospheric soil samples collected from Zawar mines, Udaipur, Rajasthan (India) in the present study.

Baliah et al. (2016) isolated phosphate solubilizing bacteria (PSB) from rhizospheric soil of different crops such as okra, chilli, tomato, cotton and eggplant and found that a total of 10 PSB strains produced a zone of solubilization around their colonies on Katznelson & Bose (KNB) solid medium. They reported that the clear or halo zone were formed due to the solubilization of insoluble phosphates by acidification of the medium. A total of 96 isolates formed halo zone in the present study might have followed the similar mechanism for formation of halo zone.

5.3. Solubilization Efficiency and Solubilization Index

Solubilization efficiency (SE) and solubilization index (SI) have been used as a screening tool for selection of potential phosphate solubilizing isolates. They provide a primary quantitative measure for the extent of solubilization of insoluble inorganic phosphate that a culture can induce (Chen, 2006). The SE for a total of 96 isolates in the present study ranged from 6.25 to 350 whereas their SI values varied between 1.02 to 4.5. Onyia et al. (2013) reported solubilization efficiencies of 12 fungal strains isolated from rhizosphere of Nsukka pepper plant. The SE values of the fungal strains varied from 109.1 to 240. Alam et al. (2002) studied the SI value for 10 bacterial strains isolated from maize rhizosphere and they reported that the SI ranged between 1.63 to 3.29. The findings of the present study are in agreement with the report of Onyia et al. and Alam et al. as a wide range of SE and SI was observed for 96 phosphate solubilizing bacterial isolates.

Ponmurugan and Gopi (2006) explained the variation in the SE and SI values. They suggested that the difference in the solubilization strength of the isolates might be attributed to microbial population size, pH and soil enzyme activity. The variation in SE and SI values for the 96 isolates in the present study can be explained on the basis of the above mentioned reason.
5.4. Tolerance of phosphate solubilizing bacteria to zinc sulphate heptahydrate

Heavy metal supplemented nutrient agar is used to determine heavy metal tolerance in bacteria. In the present study, a total of 38 out of 96 phosphate solubilizing isolates, were able to grow on nutrient agar supplemented with 1mg/ml zinc sulphate heptahydrate and were found to be zinc tolerant. Several workers (Gupta et al., 2015; Owolabi and Hekeu, 2015) have demonstrated the heavy metal tolerant ability of bacteria in nutrient agar. They explained that addition of heavy metal into nutrient agar creates selective pressure on the microorganisms for the emergence of a few strains with resistance to the heavy metals. Pal et al. (2005) stated that microorganisms isolated from natural environments contaminated with heavy metals often exhibit tolerance to metal pollutants because they have adapted to such environments. This might be true for the present study where 38 isolates (out of 96) found to be zinc tolerant were recovered from the rhizospheric soil samples contaminated with heavy metals which were collected from tailing dam of Zawar mines.

5.5. Minimum inhibitory concentration (MIC) of phosphate solubilizing bacteria to zinc sulphate heptahydrate

Minimum inhibitory concentration (MIC) is the lowest concentration of the heavy metal required to inhibit the growth of microorganisms. In the present study, MIC for 38 phosphate solubilizing bacteria to zinc sulphate heptahydrate was ranged from 2mg/ml to 24mg/ml. PSB 16 exhibited highest MIC 24mg/ml followed by PSB 11 (21mg/ml) and PSB 51 (15mg/ml). The three isolates tolerate very high concentrations of zinc. Rajkumar et al. (2008) reported 0.7 mg/ml MIC against zinc by Bacillus weihenstephanensis SM3 in their studies. Wani et al. (2014) studied the MIC of zinc for bacteria isolated from industrial area of Lagos and reported that PA6 and PA3 exhibited MIC 0.8 mg/ml. In this regard, the MIC values obtained for the three isolates PSB 11, PSB 16 and PSB 51 in the present study are far better as compared to the above mentioned reports. They (Wani et
al., 2014; Rajkumar et al., 2008) suggested that the isolates may develop metal resistance systems in an attempt to protect sensitive cellular components. Nies (1999) reviewed the metal tolerance ability of microorganisms and suggested different mechanisms which are followed by the microbes to tolerate high concentrations of heavy metals. These mechanisms include exclusion of metal ions, extra cellular precipitation, binding of metal ions to the outer surface of bacteria, enzymatic transformation, precipitation by oxidation/reduction reaction and biosynthesis of metal binding proteins or extracellular polymers. The three phosphate solubilizing isolates in the present study might have followed one of these mechanisms for tolerating high concentrations of zinc sulphate heptahydrate.

5.6. Cultural and morphological characterization of phosphate solubilizing bacteria

Bacterial colony characteristics and morphology play primary role in process of identification of the microorganism. The cultural characteristics of the five phosphate solubilizing bacterial isolates namely PSB 11, PSB 16, PSB 51, PSB 55 and PSB 91 (selected on the basis of maximum solubilization efficiency and fairly high zinc tolerance ability) were studied and a total of 3 different types of bacterial colonies were recovered on nutrient agar. PSB 11, PSB 16, PSB 51 showed medium-sized, red-brown, irregular, mucoid, flat colonies. PSB 55 showed small, yellow, circular, mucoid, entire, raised colonies; whereas PSB 91 showed off-white, circular, watery, irregular, raised colonies.

Morphological characterization of the five phosphate solubilizing isolates was done and all the isolates were found to be rod shaped, gram-negative and positive for 3% KOH reaction. Goldstein et al. (1999) studied the mineral phosphate solubilization (MPS) activity of bacterial population isolated from the roots of Helianthus annus jaegeri growing at the edge of an alkaline dry lake in the Mojave Desert. They reported that gram-negative bacteria mobilize insoluble phosphate most efficiently, by producing gluconic and 2- ketogluconic acid during the extracellular oxidation of glucose catalyzed by quinoprotein glucose dehydrogenase. The finding of the present study are in accordance with the above
report as all the five potent phosphate solubilizing bacterial isolates are found to be gram-negative.

5.7. **Biochemical characterization of phosphate solubilizing bacteria**

Biochemical tests are based on the differences in the biochemical activities of various bacteria and are used for identification of the diverse bacteria upto genus or species level. In the present study, all the five phosphate solubilizing bacterial isolates namely PSB 11, PSB 16, PSB 51, PSB 55 and PSB 91 were characterized biochemically. All five isolates were found to be catalase positive. Mbai (2012) reported biochemical characteristics for 73 bacterial isolates obtained from the root samples collected from research fields in Mwea and Ahero, Kenya and they reported all the isolates as catalase positive. They suggested that catalase is an important aspect required by the bacteria which provide protection against toxic free radicals that are generated particularly under environmental stresses. Hence, they could promote plant growth via an indirect way. The catalase positive nature of all five phosphate solubilizing bacterial isolates in the present study are in conformity with the above mentioned fact.

A total of four out of five isolates namely PSB 11, PSB 16, PSB 51 and PSB 55 were found to belong tentatively to genus *Pseudomonas*, members of family Pseudomonaceae whereas the isolate PSB 91 was primarily characterized as *Enterobacter* sp. (family Enterobacteriacea). The results of biochemical tests were in accordance with the description documented in Bergey’s manual of systematic bacteriology (Garrity et al., 2005).

*Pseudomonas* is the most commonly reported phosphate solubilizing genus (Sundara et al., 2002; Ghodsalavi et al., 2013) among all the available rhizobacteria. Midekssa et al. (2015) isolated 41 phosphate solubilizing bacterial strains from lentil rhizosphere and characterized them using biochemical tests. They reported *Pseudomonas* as the most dominant genus among the phosphate solubilizing isolates. Teshome et al. (2017) studied the isolation, identification and characterization of P-solubilizing rhizobacteria associated with *Coffea*
*arabica* L. growing in south-western Ethiopia. They found that the phosphobacteria were dominated by the genus *Pseudomonas*. The results for the present study are in agreement with the above mentioned reports as four phosphate solubilizing bacteria belonged to the genus *Pseudomonas*.

### 5.8. PIB Bryant Software Based Identification of phosphate solubilizing Bacteria

PIB Bryant software is the computer assisted identification software which is used for identification of bacteria (Bryant, 2004). PIB assisted identification was attempted using phenotypic data based on the miniaturized tests and the revised probability matrix (Carson *et al*., 2001). Willcox *et al.* (1973) developed the PIB software based on Bayes theorem which helps to calculate an identification score for the test isolates. The selection of matrix in PIB software to fed data obtained for the isolates was based upon oxidative or fermentative nature of the isolates as determined through the O/F test (section 4.8.3). The gram-negative aerobic non-fermentative (GNANFROD) matrix was used for the isolates namely PSB 11, PSB 16, PSB 51 and PSB 55 whereas gram-negative aerobic fermentative matrix (GNAFROD) was used for isolate PSB 91. The PIB score of 3 isolates (PSB 11, PSB16 and PSB 51) reached the ID score of 0.99 which is higher than the thresholds value (0.95) hence the three isolates were tentatively identified as *Pseudomonas aeruginosa*. The identification score (ID) of PSB 55 in PIB software was 0.99022 and it was tentatively identified as *Pseudomonas mandocinii* whereas the score for PSB 91 was 0.78938 which didn’t reach the threshold and the most likely taxon suggested was *Enterobacter sakazakii*.

Pai *et al.* (2011) had reported the identification of many species of *Pseudomonas* by using PIB win software. They obtained maximum identification score of 0.80411. In this regard, our results are better because high identification score was obtained in the present study for identification of *Pseudomonas* using PIB software.
5.9. Molecular characterization of phosphate solubilizing bacteria

Molecular approach based on the 16S ribosomal RNA gene is an important tool for identification of bacteria. The 16S rRNA gene is found essentially in all the prokaryotes and is mostly conserved. The universality of the genes makes them an ideal target for phylogenetic studies and taxonomic classification (Woese, 1987). However, presence of certain hypervariable regions in these 16S rRNA genes provide help to distinguish between taxa (Noller, 1984). Targeting conserved regions of the 16S rRNA gene and amplify variable regions can provide sufficient information for identification (Monstein et al., 2001).

In the present investigation molecular characterization of the five isolates was done. The genomic DNA was extracted directly from the bacteria following the method of Neumann et al. (1992). The DNA was amplified using universal primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’CGGTTACCTTGTTACGACTT-3’) designed by Weisberg et al. (1991). The amplified products (1.5kb) of all the five bacteria were submitted to Bangalore Genei Pvt. Ltd., Bangalore (India) for sequencing. The partial sequenced genes of the isolates were compared with available standard sequences of bacterial lineages in the NCBI Genbank using nBLAST. The three phosphate solubilizing isolates PSB 11, PSB 16 and PSB 51 showed 99% similarity to Pseudomonas aeruginosa therefore these three isolates were identified as Pseudomonas aeruginosa PSB 11, P. aeruginosa PSB 16 and P. aeruginosa PSB 51. Isolate PSB 55 showed 99% similarity to Pseudomonas oryzihabitans and isolate PSB 91 showed 98% similarity to Cronobacter universalis. Therefore, isolate PSB 55 was identified as Pseudomonas oryzihabitans PSB 55 and isolate PSB 91 was identified as Cronobacter universalis PSB 91. Molecular characterization of zinc tolerant PGPR strain ZN3, isolated from agricultural fields of Faisalabad, Pakistan was done by Islam et al. (2014) using universal primers 27F and 1492R and they identified the strain as Pseudomonas aeruginosa. These findings are in accordance with the findings of the present investigation.
Databases of commercially available identification systems have a considerable degree of accuracy for common species resulting in difficulties in identifying atypical strains and infrequently isolated species (Janda and Abbott, 2002). In the present study, the two isolates PSB 55 and PSB 91 were identified as *Pseudomonas mandocinii* and *Enterobacter sakazakii* by biochemical aided PIB software while they were confirmed as *Pseudomonas oryzihabitans* and *Cronobacter universalis* respectively by molecular characterization. In few instances, 16S rRNA sequencing and conventional identification by softwares based on biochemical characteristics gave different results. Woo *et al.*, (2000) reported the difference between identification system based on biochemical test and 16S rRNA analysis. They isolated a bacterial strain from the stool of a bone marrow transplant recipient suffering with diarrhea and reported that the isolate was identified as *Salmonella arizonae* (73%) by the Vitek (GNI+) systems but later 16S rRNA sequencing revealed it as *Escherichia coli*. The results of the present study are in total agreement with the above report as the identification of the two isolates PSB 55 and PSB 91 was found to be different by biochemical method as compared to that of molecular means.

The reasons for the limitations in biochemical identification may be numerous: the determination of the sole phenotypic features (which constitutes only part of the polyphasic taxonomic approach recommended to identify microorganisms), the gram-negative orientated matrix (PIB, API 20NE and BIOLOG GN2 systems) that may hinder the identification of isolates, the number of tests in the strips, and the limits of the databases content (Alatossava and Alatossava, 2006).

### 5.10. Determination of plant growth promoting activities of phosphate solubilizing isolates

#### 5.10.1. Phosphate solubilization

Phosphorus is the second key plant nutrient and is utilized by plants for their overall growth including the important metabolic processes such as cell division and development, energy transport (ATP, ADP), signal transduction, macromolecular biosynthesis, photosynthesis and respiration. The concentration
of soluble forms of P in soil is usually 1 mg/kg or even less (Goldstein, 1994). In addition to this, P has a very limited bioavailability for growing plants due to high reactivity of phosphate ions in soils. The use of phosphate solubilizing bacteria provide a suitable solution to solubilize inorganic phosphate in the soil and make it available to the plants.

In the present study, the amount of phosphate solubilized by 5 potential phosphate solubilizing bacteria namely *Pseudomonas aeruginosa* PSB 11, *P. aeruginosa* PSB16, *P. aeruginosa* PSB 51, *P. oryzihabitans* PSB 55 and *Cronobacter universalis* PSB 91 in PVK broth medium containing 0.5% TCP with pH 7 at 37°C and 180 rpm after 10 days of incubation was ranged between 90-233µg/ml. The pH of the medium dropped from initial 7 to final pH ranging between 3.2 and 4.8. El-Azeem *et al.* (2007) studied phosphate solubilization of rhizospheric bacteria isolated from Suez Canal region, Egypt. They reported that 81 isolates solubilized tricalcium phosphate (1.53 to 362.05µg/ml) in NBRIP broth after 10 days of incubation along with a reduction in pH of the medium (ranging between 6.45 to 4.16). Midekssa *et al.* (2016) studied the phosphate solubilization ability of bacteria isolated from chickpea rhizosphere. They reported that the amount of phosphate solubilization by ten bacterial isolates in PVK broth supplemented with TCP was ranged between 137 to 379 µg/ml with a drop in pH of the culture medium (ranging between 6.9 to 4.1). Both of the researchers explained that the drop in the pH was due to production of various organic acids after utilization of sugars by the isolates which finally resulted in solubilization of inorganic phosphate. The present results are consistent with the above mentioned reports. The five phosphate solubilizing bacteria in the present study might have followed a similar mechanism for solubilization of phosphate with a drop in pH of the medium as all five isolates solubilized phosphate and drop in pH of the medium was observed.

5.9.2 IAA production

PGPR are known to synthesize and release many plant growth regulators like auxins, cytokinins, gibberellins etc. Auxins are secondary metabolite produced by rhizobacteria in response to the rich supplies of substrates exuded from the roots.
Indole acetic acid (IAA), the most important native auxin, plays an important role in regulating cell division, cell elongation, cell differentiation and pattern formation in plants. These compounds stimulate root growth and increase root length, resulting in a larger root surface area that enables the plant to access more nutrients from the soil (Souza et al., 2013). In natural conditions, the microbial biosynthesis of IAA in soil is enhanced by tryptophan from root exudates or decaying cells. The application of organic fertilizer can increase the levels of tryptophan in soil and tryptophan found in organic wastes and fertilizers may be produced by aerobic or anaerobic microbial transformation (Arkhipchenko et al., 2006).

The in vitro production of IAA in the present study by five phosphate solubilizing bacteria namely Pseudomonas aeruginosa PSB 11, P. aeruginosa PSB16, P. aeruginosa PSB 51, P. oryizihabitans PSB 55 and Cronobacter universalis PSB 91 without addition of tryptophan to the nutrient broth at 37°C and 180 rpm after 48 h was found to range between 5.75 to 9.24µg/ml. On addition of 1000µg/ml of L-tryptophan to the nutrient broth the quantity of IAA produced by the isolates increased and was ranged between 24.3 to 35.5µg/ml.

Khalid et al. (2004) studied the in vitro production of IAA in absence as well as presence of L-tryptophan by thirty bacterial isolates recovered from wheat rhizosphere. They found that bacterial efficiency for indole production enhanced several folds on addition of tryptophan. They suggested that L-tryptophan is likely to stimulate auxin biosynthesis by acting as a precursor for IAA production. Our results are in agreement with the work of Khalid et al. as incorporation of L-tryptophan to the medium enhanced in vitro production of IAA by all five phosphate solubilizing isolates.

Patten and Glick (1996) reported that the variation in the amount of IAA produced by different isolates is attributed to the difference in their genetic makeup, growth kinetics, biosynthetic pathways, location of the genes involved, regulatory sequences, and the presence of enzymes which convert active free IAA into conjugated forms. This report might provide a suitable explanation for the
variation in the amount of IAA produced by the five phosphate solubilizing isolates in the present study.

5.9.3 Siderophore production

Siderophores are small peptidic molecules containing side chains and functional groups that can provide a high-affinity set of ligands to coordinate ferric ions. Iron exists in an insoluble form (Fe$^{3+}$) in the soil which is not easily available to the plants and microbes. To gain access to such unavailable iron sources, most bacteria produce ferric iron-chelating compounds known as siderophores. PGPR act as biocontrol agent against the plant pathogens by producing siderophores that chelate the available iron and deprive the iron nutrition for respective phytopathogens. They also improve plant growth by providing iron. Plants can assimilate iron from bacterial siderophores by means of different mechanisms, for instance, chelate and release of iron, the direct uptake of siderophore-Fe complexes, or by a ligand exchange reaction (Schmidt, 1999; Crowley and Kraemer, 2007).

In the present study, all five phosphate solubilizing bacteria namely *Pseudomonas aeruginosa* PSB 11, *P. aeruginosa* PSB 16, *P. aeruginosa* PSB 51, *P. oryzihabitans* PSB 55 and *Cronobacter universalis* PSB 91 produced siderophore efficiently both on solid Chrome azurol S (CAS) agar and in modified Fiss minimal medium. The diameter of the orange-yellow halo zone for the five isolates on blue coloured CAS agar at 37°C after 5 days of incubation ranged between 10 to 18 mm. The siderophores units produced by five phosphate solubilizing bacteria in modified Fiss minimal medium at 37°C in incubator shaker at 180 rpm after 5 days ranged between 37.82 to 71.35%.

Sharma *et al.* (2016) studied the siderophore production using (CAS) agar plates by eight bacterial strains isolated from Tarai region of Uttarakhand, India. They reported that only three isolates were found to produce more than 60% siderophore units (SU). The isolate PB19 was found to be most efficient, produced 78% SU and was identified as *Pseudomonas* spp. Tailor *et al.* (2012) screened the siderophore producing bacteria from the sugarcane rhizosphere by
CAS agar method. They reported that seven isolates produced more than 85% siderophore units. *Pseudomonas fluorescens* was found to be the most efficient siderophore producer (96% SU). Both the researchers used the universal assay of Schwyn and Neilands (1987) for siderophore detection using CAS dye. This assay is based on a competition for iron between the ferric complex of the dye chrome azurol S (CAS) and a siderophore. The CAS dye complexes tightly with ferric iron to produce a blue colour. When siderophore, a strong iron chelator, removes iron from the dye complex, the color changes from blue to orange. The results for the present study are in accordance with the above observations as orange-yellow halo zone were produced by the five phosphate solubilizing isolates on blue coloured CAS agar and might have followed a similar mechanism for siderophore production.

**5.9.4 HCN production**

HCN production by rhizobacteria is a potential and environmentally compatible mechanism for biologically controlling weeds and minimizing deleterious effects on the growth of host plants. HCN is a secondary metabolite and can help plants in their defense against phytopathogens. Cyanide acts as a general metabolic inhibitor to avoid predation or competition. It inhibits cytochrome- C oxidase efficiently along with several other metalloenzymes. However, the host plant is not being affected by the negative action of cyanide with respect to root development and root metabolism (Schippers *et al.*, 1987; Saharan and Nehra 2011).

In the present study, a total of three out of five phosphate solubilizing bacteria namely *Pseudomonas aeruginosa* PSB 11, *P. aeruginosa* PSB 16, *P. aeruginosa* PSB 51, were found to produce HCN on tryptic soya agar (TSA) medium amended with 4.4 g/l glycine when incubated at 37°C for 48 h. The HCN production was confirmed with a change in colour of the filter paper from yellow to orange-brown. Fauzia *et al.* (2015) studied plant growth promoting activities of three fluorescent pseudomonads isolated from the wheat rhizosphere. They reported the appearance of light brown to dark brown color of filter paper which indicated HCN production by all the tested strains. The results for the present
study are in agreement with the above observations as production of HCN was detected through change in colour of the filter paper from yellow to orange-brown by three of the phosphate solubilizing bacterial isolates. Ramette et al. (2003) studied the phylogeny of \textit{hcncB} genes encoding the HCN synthetase enzyme and reported that the formation of HCN from glycine was catalyzed by a membrane flavoprotein enzyme HCN synthetase. Three of the phosphate solubilizing bacterial isolates in the present study might have followed the similar mechanism for HCN production.

5.9.5 Ammonia production

Ammonification is an important step in the transformation of organic nitrogen to ammoniacal form which can enhance soil nitrogen content by the ammonifying character of the PGPR isolates (Dey et al., 2004). Therefore, involvement of ammonification trait of the PGPR strains might be significant in improving plant growth. In the present study, all five phosphate solubilizing bacterial strains namely \textit{Pseudomonas aeruginosa} PSB 11, \textit{P. aeruginosa} PSB16, \textit{P. aeruginosa} PSB 51, \textit{P. oryzihabitans} PSB 55 and \textit{Cronobacter universalis} PSB 91 were found positive for production of ammonia after incubation at 37°C for 48 h. All the isolates showed a change in colour from yellow to dark orange on addition of Nessler’s reagent in test tubes containing inoculated and incubated media.

Agbodjato et al. (2015) studied the plant growth promoting characteristics of sixty bacteria isolated from maize rhizosphere and found that all the species of \textit{Serretia} were capable of producing ammonia. Wani et al. (2014) studied the plant growth promoting activities of metal tolerant isolates PA1 to PA6 and reported that all six strains produced ammonia efficiently. They suggested that the production of ammonia is essential in plant growth promotion as it play a signalling role during interaction with host plant. All five isolates in the present study have produced ammonia and can be further explored for promotion of plant growth.
5.11. Effect of various factors on phosphate solubilization by phosphate solubilizing bacteria

Phosphate solubilization is a biochemical phenomenon carried out by numerous rhizobacteria through different mechanisms depending upon the environmental conditions. The growth and survival of phosphate solubilizing bacteria is affected by various physico-chemical conditions which in turn may affect the mechanisms of phosphate solubilization. Since the rhizobacteria resides in soil surrounding the root of plant, the process can be affected by the soil environment. Hence, there is a need to understand the effect of various factors such as media, temperature, pH and presence of some heavy metals on phosphate solubilization so that the isolates can be best utilized accordingly for improving the growth of plants in given environmental conditions.

5.11.1. Effect of media

The solubilization ability of phosphate solubilizing bacteria is usually studied by growing them in media containing inorganic phosphate compounds as the sole phosphate source such as tricalcium, iron and aluminium phosphate, hydroxyapatite, bone meal, rock phosphate etc. However, other components of the media also interfere in the activity of phosphate solubilization. In the present study three different media namely Pikovskaya’s (PVK) broth, National Botanical Research Institute’s phosphate broth medium (NBRIP) and modified Sperber’s broth were used to determine the effect of media on the amount of phosphate solubilized by five phosphate solubilizing bacteria namely *Pseudomonas aeruginosa* PSB 11, *P. aeruginosa* PSB16, *P. aeruginosa* PSB 51, *P. oryzihabitans* PSB 55 and *Cronobacter universalis* PSB 91. A considerable amount of phosphate was solubilized by five isolates in all the three culture media used in the study. Among the three culture media used, modified Sperber’s medium was found to be the best for phosphate solubilization by all five isolates at 37°C and 180 rpm after 10 days of incubation. Among the remaining two media (NBRIP and PVK medium) used, the amount of phosphate solubilized by all the five isolates in NBRIP medium was more as compared to that in PVK medium.
Son et al (2006) isolated Pantoea agglomerans R-42 from rhizosphere of soyabean plants growing in fields of Miryang, Korea. They studied the effect of insoluble phosphate source on solubilization capacity of the isolate by supplementing modified PVK medium with five different insoluble phosphates (TCP, CaHPO$_4$, FePO$_4$, AlPO$_4$ and hydroxyapatite). They reported that the production of soluble phosphate by P. agglomerans with TCP, CaHPO$_4$ and hydroxyapatite was significantly higher as compared to FePO$_4$ and AlPO$_4$. They further reported that the amount of phosphate solubilized by the isolate from CaHPO$_4$, hydroxyapatite and TCP was 1367mg/l, 1357mg/l and 1312mg/l respectively. They suggested that the isolate Pantoea agglomerans R-42 has the potential to solubilize hydroxyapatite form of insoluble phosphate. In this regard, all five phosphate solubilizing bacterial isolates in the present study showed their ability to solubilize hydroxyapatite more efficiently than tri calcium phosphate as indicated by maximum phosphate solubilization by all the isolates in modified Sperber’s medium (containing hydroxyapatite).

Nautiyal (1999) studied the phosphate solubilization ability of eight Pseudomonas strains isolated from the soil and roots of plants growing in fields at Banthra village, Lucknow, India in two different media namely PVK and NBRIP broth media. They found that the solubilization in NBRIP broth was about 3-fold more as compared to that in PVK broth for all eight strains. The results of the present study are in agreement to the above mentioned findings. Nautiyal explained that because of the difference in composition of these two media the amount of phosphate solubilized varied. He suggested that although, the source of inorganic phosphate was same for two media i.e., 0.5% TCP but yeast extract was not essential in the medium for phosphate solubilization whereas glucose and magnesium chloride are most essential for the activity. This explanation may be given for more phosphate solubilized in NBRIP medium as compared to that in PVK in the present study.

5.11.2. Effect of temperature

Temperature is a vital factor for the growth and activity of microorganisms (Gaind and Gaur, 1990). The effect of temperature on phosphate solubilization by all five
phosphate solubilizing bacteria namely *Pseudomonas aeruginosa* PSB 11, *P. aeruginosa* PSB16, *P. aeruginosa* PSB 51, *P. oryzihabitans* PSB 55 and *Cronobacter universalis* PSB 91 was determined in modified Sperber’s medium at three different incubation temperatures i.e., 28, 37 and 42°C. All five isolates showed maximum phosphate solubilization in modified Sperber’s medium at 28°C incubation temperature after 10 days of incubation.

Malboobi *et al.* (2009) studied the phosphate solubilization for three rhizospheric bacteria namely *Pantoea agglomerans*, *Microbacterium laevaniformans* and *Pseudomonas putida* in Sperber’s medium at three different temperatures 25, 35 and 42°C. They reported that maximum solubilization for all three isolates was at 25°C. They also suggested that bacterial growth and consequently phosphate solubilization of bacteria were reduced at higher temperatures. Gaur (1990) also reported that a range of temperature between 25 and 30°C was found to be most suitable for phosphate solubilization by strains of *Pseudomonas striata*. The findings of the present study are in close conformity with the above reports as maximum phosphate solubilization for all five phosphate solubilization bacterial isolates was obtained at 28°C.

Some other workers have reported maximum phosphate solubilization at comparatively high temperatures (Nautiyal *et al.*, 2000; Gaind and Gaur, 1991). Nautiyal *et al.* (2000) isolated four phosphate solubilizing bacteria NBRI0603, NBRI2601, NBRI3246 and NBRI4003 from the rhizosphere of chickpea and alkaline soils from Banthara Research Station, Lucknow, India. They studied the effect of temperature on the solubilization of phosphate by all the strains in NBRIP medium and found that NBRI2601 strain proved to be the most efficient strain in solubilizing phosphate at 45°C. Gaind and Gaur (1991) isolated phosphate solubilizing microorganisms from leguminous (cowpea, green gram, groundnut, pigeon pea) and non-leguminous crops (wheat, oat, maize) and from samples of compost, farmyard manure, Mussoorie rock phosphate and garden soil amended with 5% rock phosphate. They studied the efficiency of phosphate solubilizing microorganisms at three temperatures viz. 35, 40 and 45°C. They reported that the isolates solubilized TCP most efficiently at 45°C among the three
temperatures. These observations clearly suggest that bacteria adapt to their indigenous environment, so their metabolic activities are linked to the temperature of the environment (Shahab and Ahmed, 2008) and hence they respond accordingly.

The findings of the present study are in contradiction with the reports of Nautiyal et al and Gaind and Gaur. In the present study all five phosphate solubilizing bacterial strains might have adapted to 28 °C and therefore shown maximum phosphate solubilization in modified Sperber’s medium at this incubation temperature.

5.11.3. Effect of initial pH
Soil pH is one of the most important factors affecting the availability of inorganic phosphate to plants. The pH of the medium is known to affect the growth and activity of the microorganisms (Gaind et al., 1990). All five isolates namely Pseudomonas aeruginosa PSB 11, P. aeruginosa PSB16, P. aeruginosa PSB 51, Pseudomonas oryzihabitans PSB 55 and Cronobacter universalis PSB 91 were analyzed for phosphate solubilization activity in modified Sperber’s medium with three different initial pH values viz., 4, 7, 9. All five isolates showed phosphate solubilization in modified Sperber’s medium with different initial pH values. Maximum phosphate solubilization was obtained in modified Sperber’s medium with initial pH 7 when incubated at 28°C with 180 rpm for 10 days by all five isolates.

Walpola et al. (2014) studied the effect of wide range of pH (4-10) on phosphate solubilizing ability of Klebsiella oxytoca isolated from abandoned mines at Boryeong area in South Korea. They found that the maximum phosphate solubilization by the isolate was obtained in NBRIP medium with initial pH 7. Rathore et al., (2012) isolated Citrobacter freundii from agricultural soils of Indore. They studied the effects of different initial pH values (3-9) of PVK medium on phosphate solubilization by the isolate. They reported that the maximum solubilization of phosphate occurs in medium with pH 7. They explained that the organism seems to have better growth at the neutral pH. As the growth increased acid production in the medium also might increase bringing the
pH to lower acidic side and thereby increasing solubilization with time. Initial acidic pH (or the alkaline pH) may not allow the efficient growth of the organism thereby affecting both acid production and phosphate solubilization. The present results are in conformity with the above reports (Walpola et al. and Rathore et al.) as the maximum solubilization of phosphate by all five isolates was obtained in modified Sperber’s medium with initial pH 7. The possible reason for maximum phosphate solubilization by all five isolates in modified Sperber’s medium with initial pH 7 in present study could be explained by the mechanism described by Rathore et al.

However Nautiyal et al. (2000) demonstrated that phosphate solubilization can take place in culture medium with high initial pH value. They studied the phosphate solubilization by four strains namely NBRI0603, NBRI2601, NBRI3246 and NBRI4003 in NBRIP medium with diverse level of pH values. They reported that phosphate solubilization abilities of the four strains were higher than the control in the medium with pH 8. They explained that since the strains were isolated from alkaline soils so they have the potential to solubilize phosphates at high pH. In contrast to this, the bacterial strains in the present study showed maximum phosphate solubilization in modified Sperber’s medium with initial pH 7. This might be because these strains were isolated from soil with neutral pH.

5.11.4. Effect of zinc sulphate heptahydrate

Microbial phosphate solubilization depends upon environmental factors and gets severely affected under stress conditions like presence of heavy metals. Toxic levels of heavy metals including zinc in the soil restrict the growth of both plants and associated bacteria. High concentration of zinc alters the cellular metabolism of microbes and affects their physiological activities. The present investigation involves the study of effect of varying concentrations (4, 8, 12mg/ml) of zinc sulphate heptahydrate on phosphate solubilizing activity by three phosphate solubilizing bacterial isolates namely Pseudomonas aeruginosa PSB 11, P. aeruginosa PSB16 and P. aeruginosa PSB 51 (which were found zinc tolerant in Section 4.5) in modified Sperber’s medium (pH7) at 37°C and 180 rpm after 10
days of incubation. A gradual decrease in phosphate solubilization was recorded with increasing concentrations of ZnSO₄·7H₂O to modified Sperber’s medium for all the three phosphate solubilizing bacterial isolates. All three bacterial strains solubilized phosphate to some extent at 4, 8, 12mg/ml concentrations of ZnSO₄·7H₂O in modified Sperber’s medium.

Misra et al. (2012) isolated six bacterial strains namely *Pseudomonas aeruginosa* RS7, *Serratia spp.* EPR1, *Enterobacter cloacae* RP11, *Serratia spp.* RS5, *Acinetobacter haemolyticus* RP19 and *Citrobacter freundii* RP23 from the rhizospheric soil of *Achyranthes aspera* collected from Hindustan Zinc Ltd., Udaipur, India. They studied the effect of zinc sulphate on the phosphate solubilizing activity of all six isolates in NBRIP broth medium. They reported that addition of ZnSO₄ (0.3–1.5 M) in the medium resulted in 30–73% inhibition of the phosphate solubilization activity by all six isolates. The results of the present study are in accordance with the findings of Misra et al. where addition of varying concentrations of zinc sulphate heptahydrate to modified Sperber’s medium resulted in decrease (29.16 to 66.95%) in the amount of phosphate solubilized by three isolates.

Bolan et al. (2008) described reduction in solubilization of phosphate by means of phosphate-induced immobilization of metals. Various mechanisms including direct metal adsorption by phosphate compounds, phosphate anion-induced metal adsorption, direct precipitation of metals with phosphate in solution as metal phosphates, precipitation through the liming action of rock phosphate, and rhizosphere modification through acidification have been proposed which results in the unavailability of phosphate to microbes. The reduction in phosphate solubilization by the three isolates in the present study can be explained on the basis of phosphate-induced immobilization of metals as described in the above mentioned report.
5.12. Effect of application of phosphate solubilizing bacteria on the growth of *Triticum aestivum* L. grown in soil supplemented with tricalcium phosphate

Plant growth promotion by rhizobacteria is a complex phenomenon which involves the contribution of more than one plant growth promoting trait such as phosphate solubilization along with production of IAA, siderophore, HCN and ammonia. It is a well-established fact that improved phosphorus nutrition influences overall plant growth and root development (Jones and Darrah, 1994). A large number of researchers have demonstrated that PSB strains could improve growth in plants, such as *Triticum aestivum* L. (Mukherjee and Rai, 2000), *Zea mays* L. (Nadeem et al., 2007), *Brassica juncea* (Kumar et al., 2009), *Aloe barbadensis* (Gupta et al., 2010) and sugarcane (Beneduzi et al., 2013).

In the present investigation, effect of five phosphate solubilizing bacteria namely *Pseudomonas aeruginosa* PSB 11, *P. aeruginosa* PSB16, *P. aeruginosa* PSB 51, *P. oryzihabitans* PSB 55 and *Cronobacter universalis* PSB 91 on growth of *Triticum aestivum* L. in tricalcium phosphate supplemented soil under potted conditions was studied and observations were recorded after 45 days. A significant increase in all the growth parameters (shoot length, root length, shoot fresh weight, shoot dry weight, root fresh weight) of *Triticum aestivum* L. was observed in T1 (soil supplemented with TCP) treatment as compared to T0 (control). Inoculation of all five phosphate solubilizing bacteria separately resulted in increase in all the growth parameters including shoot length, root length, shoot fresh weight, root fresh weight, shoot dry weight and root dry weight of *Triticum aestivum* L. plants except root dry weight which was found to be similar (for isolates *P. aeruginosa* PSB16 and *Cronobacter universalis* PSB 91) to that observed in T1 treatment.

The overall increase in growth of the wheat plant in T1 treatment may be because of addition of TCP (200mg/kg) to soils as compared to T0 (6.51mg/kg). Nwanyanwu et al. (2015) studied the effect of phosphate solubilizing bacterial
strains (Pseudomonas sp. PSBA2, Pseudomonas sp. PSBN1 and Bacillus sp. PSBC1) on shoot length of maize seedlings in potted soil. They reported maximum increase in shoot length of maize seedlings when inoculated with Pseudomonas sp. PSBN1. They suggested that the increase in shoot length could be associated with cell elongation and multiplication induced by greater absorption of nutrients, particularly phosphorous (by phosphate solubilizing bacteria which solubilizes the unavailable form of phosphate and make it available to maize plant). The results for the present study are in complete agreement with the report of Nwanyanwu et al. as the shoot length of the wheat plant showed a significant increase on inoculation of all five bacterial strains separately. The five phosphate solubilizing isolates in the current study might have followed the mechanism described in above report which resulted in increase in shoot length of wheat plant on application of phosphate solubilizing bacteria. Jha et al. (2012) isolated four phosphate-solubilizing bacterial strains viz. Pseudomonas fluorescens BAM-4, Burkholderia cepacia BAM-6, B. cepacia BAM-12 and Aeromonas vaga BAM-77 from the rhizosphere of pearl millet, mung bean and sesame. They studied the effect of seed inoculation of single and dual bacterial cultures on mung bean in pot trials having sterilized sandy loam soil supplemented with (0.2%) or without tricalcium phosphate (TCP). They reported increase in shoot dry weight and suggested that the increase in shoot dry weight can be due to the additional availability of phosphates to the plants. The results for the present study are in accordance with the report of Jha et al as the shoot dry weight of the wheat plant showed a significant increase on inoculation of five bacterial strains separately. The increase in the shoot dry weight of wheat plants as reported in the current study might be due to the additional availability of phosphates solubilized by the five phosphate solubilizing isolates in the soil.

Islam et al. (2014) studied the effect of inoculation of Pseudomonas aeruginosa ZN3 on the root length of wheat plant. They reported an increase in the root length of wheat plant on treatment with the bacterial strain. They suggested that IAA production by the isolate improved the lateral and adventitious root growth of the wheat plant which enhanced the mineral and nutrient uptake. The results for the present study are in accordance with the above mentioned report as
inoculation of wheat seeds with all five phosphate solubilizing bacterial strains separately showed increase in the root length of the wheat plants. All five phosphate solubilizing bacterial isolates produced IAA \textit{in vitro} as reported in section 4.10.2. These isolates might have produced IAA in the soil which might have resulted in increase in the root length.

5.13. Effect of application of phosphate solubilizing bacteria on the growth of \textit{Triticum aestivum} L. grown in soil supplemented with tricalcium phosphate in presence of zinc sulphate heptahydrate

Plant growth promoting bacteria, exhibiting metal tolerance, provides an excellent approach for augmenting the growth of plants growing in metal contaminated soil. In the present study, the effect of three phosphate solubilizing bacterial isolates namely \textit{Pseudomonas aeruginosa} PSB 11, \textit{P. aeruginosa} PSB16, \textit{P. aeruginosa} PSB 51 (which were found zinc tolerant as described in section 4.5) on the growth of wheat (\textit{Triticum aestivum} var. RAJ 4238) sown in soil supplemented with tricalcium phosphate (200mg/kg) in presence of zinc sulphate heptahydrate was evaluated and observations were recorded after 45 days. On addition of 1000 mg/kg zinc sulphate heptahydrate to soil (TZ1), decrease in all the growth parameters (shoot length, root length, shoot fresh weight, shoot dry weight, root fresh weight and root dry weight) of wheat plant was observed as compared to T1 treatment [TCP (200mg/kg) amended soil]). Application of three bacterial strains namely \textit{Pseudomonas aeruginosa} PSB 11 (TZ2a), \textit{P. aeruginosa} PSB16 (TZ2b) and \textit{P. aeruginosa} PSB 51 (TZ2c) separately showed a remarkable increase in all the growth parameters (shoot length, root length, shoot fresh weight, shoot dry weight, root fresh weight and root dry weight) when sown in soil supplemented with tricalcium phosphate (200mg/kg) and ZnSO$_4\cdot$7H$_2$O (1000 mg/kg) as compared to TZ1 treatment. The stress tolerance index (STI) for different growth parameters for the TZ1 and TZ2 treatments were ranged between 0.38-0.74 and 1.35-3.80 respectively.
Heavy metals at higher concentration are highly phytotoxic and induce visible injuries as well as physiological and biochemical alterations in plants which results in retarded growth (Zhou et al., 2007; Islam et al., 2014). Wani et al. (2007) assessed the effect of Bradyrhizobia strain RM8 on growth (dry weight) and nodulation of green gram, grown in sandy clay loam soil amended with varying concentrations of nickel (0.145, 0.290 and 0.58 g/kg) and zinc (2.445, 4.89 and 9.78g/kg). They demonstrated a significant inhibition in plant growth and nodulation when exposed to different concentrations of nickel and zinc. Manivasagaperumal et al. (2011) studied the effect of varying concentrations (0-200mg/l) of zinc sulphate solution on germination, seedling growth, fresh and dry weight of shoot and root of cluster beans. They reported that high concentrations of zinc (50-200mg/l) reduced the seed germination, seedling length, fresh and dry weight of both shoot and root of cluster bean seedling. They reported the decrease in plant biomass in excess of zinc might be due to reduction in cell division, low protein formation, resulting in the inhibition of photosynthesis, as well as hampering of carbohydrate. The results of the present investigation are in conformity with the findings of Wani et al. and Manivasagaperumal et al. as growth of wheat plant reduced with exposure of zinc sulphate heptahydrate. The significant decrease in the growth parameters of wheat plant on addition of ZnSO₄·7H₂O to the soil in the present investigation can be attributed to any of the mechanisms explained by Manivasagaperumal et al.

Islam et al. (2014) studied the effect of inoculation of Pseudomonas aeruginosa ZN3 on the shoot length, root length and total dry weight of wheat plant under zinc stress. They reported that the shoot length, root length and total dry weight of wheat plant (under zinc stress) significantly increased on inoculation of Pseudomonas aeruginosa ZN3. The finding of the present study are in accordance with the report of Islam et al where inoculation of five phosphate solubilizing bacterial isolates separately to the wheat seeds increased all the growth parameters of wheat plant. Different heavy metal tolerance mechanisms have been reported in rhizobacteria which involves exclusion, active removal, biosorption, precipitation or bioaccumulation of heavy metals both in external and intracellular spaces. These processes can influence the solubility and the bioavailability of the metal to
the plant and thus modify the toxic effects of the metal (Haferburg and Kothe, 2007; Harrison et al., 2007). The three phosphate solubilizing bacterial strains in the present study might have followed one of the above mentioned mechanisms to reduce the toxic effect of zinc sulphate heptahydrate on wheat plant.

Burd et al. (1998) studied the effect of inoculation of Kluyvera ascorbata SUD165 on canola and tomato grown in presence of high concentrations (1 to 6 mM) of nickel (Ni\(^{+2}\)). They reported that high concentrations of nickel reduced the growth of both canola and tomato. However, addition of Kluyvera ascorbata SUD165 to canola and tomato seeds before germination significantly decreased the toxicity of the added nickel. They presented their results as tolerance index TI, where TI = \( \frac{L_m}{L_c} \) (\( L_m \) is the shoot or root length of plant grown in the presence of specific added metal and \( L_c \) is the shoot or root length of plant grown in the absence of that metal). They calculated TI for shoot and root for both canola and tomato at all concentrations of Ni\(^{+2}\) tested and after addition of K. ascorbata SUD165. They reported TI varying between 0.12-0.57 at different concentrations of Ni\(^{+2}\) which represent an inhibitory effect of nickel on plants whereas on addition of K. ascorbata SUD165 the TI increased and ranged between 0.18-0.80 for all the treatments and represented a reduced inhibitory effect of nickel. In this regard the findings of the present study are far better as compared to the above mentioned report as inoculation of three phosphate solubilizing bacterial isolates resulted in increase in stress tolerance index (STI 1.35 to 3.46) in TZ2a, b, c treatment as compared to TZ1 treatment (STI ranged between 0.38 to 0.74).

Therefore, the newly isolated bacterial strains Pseudomonas aeruginosa PSB 11, P. aeruginosa PSB 16 and P. aeruginosa PSB 51 in the present study were proved to be highly effective in protecting wheat plants from growth inhibition caused by the presence of high concentrations of zinc sulphate heptahydrate. The inoculation of these efficient PGPR strains as a biotechnological tool for reducing the zinc toxicity will help in understanding various adaptive processes which are poorly understood till date.